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FOREWORD

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TABLE OF CONTENTS

Introduction

The project to test expression of Cre recombinase directed by the *Mtv*-17 locus is in its first year of three years of funding. The hypothesis of the project was to develop a novel and useful breast cancer model using the tissue specific expression of the *Mtv*–17 locus, which has previously been shown to express only in the mammary gland. We proposed to 'knocking–in' Cre recombinase at the *Mtv*–17 env region and this should result in expression of this enzyme only in the mammary gland. Mice that carry the *Mtv*–17 Cre fusion vector will be mated to lox P p53 conditional knock–out mice, resulting in loss of functional p53 in the mammary gland only. These animals should only develop mammary tumors and not other tumors. Our knock–in vector will provide a conditional knock–out system and locus for mammary gland specific expression of many different transgenes and should be of widespread utility to the study of both transformation and development of this tissue.

At this time, the progress of the project can be divided into three parts. The first part is vector construction. The second part is production of animals. The third portion is the obtaining of certain reagents.

Vector Construction

Three vectors were described in the proposal. All three vectors are knock-in vectors. These vectors by definition are designed to place either a gene or sequence in a specific locus by homologous recombination in embryonic stem cells.

The first vector has been constructed to 'knock-in' Cre recombinase into the *Mtv*-17 locus at the env position (figure 1). This is accomplished through vector design which mutants the endogenous ATG start site of env followed by the ATG start site of Cre recombinase. The second vector is similar to the first. It is also designed to homologously recombine with the *Mtv*-17 locus (figure). However, in the second vector Cre recombinase is placed in the gag position and the ATG start site of gag is removed in the construct. Both of theses vectors have been constructed and have been used to transform embryonic stem (ES) cells.

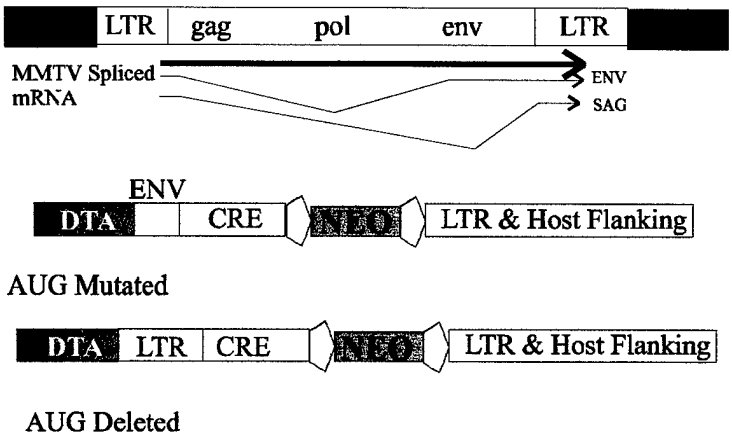

Figure 1: The two knock-in constructs which express Cre Recombinase under the direction of the *Mtv-17* locus. The top figure is the endogenous locus and the mRNA which is synthesized from that locus. The second figure is the vector which is designed to homologously recombine with env position of the locus. The bottom panel is the second vector which is designed to homologously recombine with the gag position of *Mtv*-17 locus. Long Terminal Repeats (LTR), Cre Recombinase (CRE), Neomycin resistance (NEO), Diphtheria Toxin A (DTA).

The third and final vector is designed to under go homologous recombination with the p53 locus (figure 2). Rather than knocking-in a gene such as Cre recombinase. This vector has been designed to embed lox P sites into introns four and six. The result is the production of normal p53 protein in all cells except those that express Cre recombinase. In cells that express Cre recombinase, a mutant protein will be formed which retains the tetramerization domain but lacks a DNA binding domain. This protein is predicted to be a dominant mutant. Even an animal heterozygous for the mutant p53 should have an elevated rate of cancer. The p53 knock-in vector is two steps from being finished at this time.

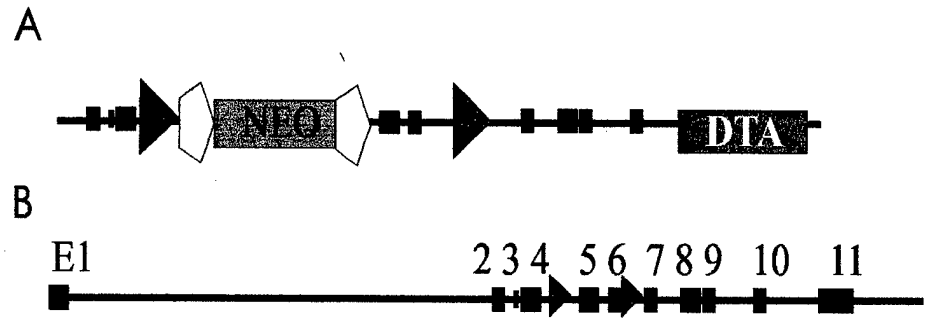

Figure 2: The p53 knock-in vector (panel A) and endogenous p53 locus following homologous recombination (panel B). The lox p sites represented by the triangles are embed in the introns, represented by the solid lines and the exons are represented by the solid boxes. (See figure 1 for abbreviations).

Animal Production

The first two targeting vectors, which knock-in Cre recombinase, have been transfected into the C57/Bl6 embryonic stems cells. To date, 197 neomycin (neo) positive colonies have been obtained using the first knock-in vector(figure 1). It is designed to place Cre in the env position of the *Mtv*-17 locus. These neo colonies were then screened by a polymerase chain reaction using primers directed against the portion of the envelope that is only in the endogenous *Mtv* loci and not in the knock-in vector. The other primer is located in Cre recombinase which is only in the targeting vector. The only way that the desired 1.5 Kb product of this PCR can be obtained is if homologous recombination at an *Mtv* locus has occurred. The PCR screen is only preliminary since a positive 1.5 Kb product could also be obtained from recombination of vector with either of two other endogenous *Mtv* loci present in the C57/Bl6 genetic background. However, this is less likely due to the large amount of host flanking region which should primarily if not exclusively direct the vector into the *Mtv*-17 position.

Four of the 197 clones produced a 1.5 Kb fragment. These clone will be expanded and tested using by southern analysis for confirmation of the correct locus. The four clones represent a possible homologous recombination to the correct locus but in no way should be considered correct until the southern analysis can be performed.

The second knock-in vector is designed to place Cre in the gag position of the *Mtv*-17 locus. Transfection into ES cells has produced 94 neo positive colonies. These colonies can not easily be screened by PCR. Rather, these colonies have been unsuccessfully been screened by southern blot analysis of the genomic DNA of the embryonic stem cells. More colonies will need to generated and tested.

The two male lox P B-gal reporter mice (a kind gift of David Anderson, Ph.D. California Institute of

Technology) were obtained. After a period of quarantine, these mice were bred to C57/Bl6 females to generate a F2 generation. The F2 animals were tested by PCR analysis for the presence of the B-gal gene and as expected all animals in the F2 generation possessed the construct. The heterozygous F2 females were bred with the F1 homozygous males to generate a F3 generation. By Mendelian genetics, 50% of the F3 offspring should be homozygous for the reporter transgene. The easiest way to test the genotype of these animals with respect to the transgene is to test by breeding. The F3 generation, once it reaches sexual maturity, will be bred to animals that are nullizygous for the transgene. The resulting offspring will be genotyped by PCR analysis. The genotype of the offspring will allow us to determine the F3 generation genotype and homozygous animals can be crossed to generate a homozygous colony.

Obtaining External Reagents

The Balb/c genomic p53 construct was a kind gift of Lawrence Donehower, Ph.D. This was used in the production of the p53 knock-in vector. The lox P reporter mice were a kind gift of David Anderson, Ph.D. These mice will be used to test the tissue specific expression of Cre Recombinase under the direction of the *Mtv*-17 locus. A Cre Recombinase expressing adenovirus was a kind gift of Frank Graham, Ph.D. The adenoviral infection of lox P mice will reveal any limitations if any of the reporter mice have in expression of β-galactosidase. The leukemia inhibitory factor (LIF) expressing CHO cells were a kind gift of the Genetics Research Institute, Cambridge, MA. The C57/ Bl6 mouse embryonic stem cells were a kind of Collin Stewart, Ph.D.

Conclusions

In summary, we have produced two of the three homologous recombination vectors proposed for the project. The third vector is nearly complete. Embryonic stem cell production is continuing with four potentially positive clones being obtained for one of the constructs. All of the reagents, which we proposed obtaining from outside sources, have been obtained. In general, the research has progressed according to the original statement of work with a slight lag in the production of the p53 vector. However, this vector is not necessary until later in the project since tissue specific expression of Cre recombinase must first be ascertained before breeding to p53 conditional knock-out mice can be done.

As I have already informed the Army, I will be leaving this project and the University of Pennsylvania to take a research and development position in industry at the end of January 1999. I strongly recommend that this project be continued in Professor Susan Ross' Laboratory with Jennifer Czarneski, Ph.D. as principal investigator. This project has great potential and has progressed according to the statement of work with all the necessary reagent to complete this project having been obtained. It is my sincere hope that the US Army Breast Cancer Research Program in its wisdom will see the benefits in continuing to fund the last two years of this project in my absence.